Specification

β-FRUCTOFURANOSIDASE VARIANTS

[Field of the Invention]

The present invention relates to β-fructofuranosidase variants which selectively and efficiently produce specific fructooligosaccharides from sucrose, and more specifically, to β-fructofuranosidase variants which efficiently produce 1-kestose and β-fructofuranosidase variants which efficiently produce nystose.

[Background Art]

Generally, fructooligosaccharides are oligosaccharides in which one to three fructose molecules are bound via β-bonds at positions C1 and C2 to the fructose moiety of sucrose and are indigestible sugars known for their excellent physiological functions, such as stimulation of the growth of bifidobacteria in the intestines, improvement in metabolism of cholesterols and other lipids, low cariogenicity, and stimulation of mineral absorption. Fructooligosaccharides are known to be widely distributed in nature in plants, such as onion, asparagus, and Jerusalem artichoke. Since technology for the mass production from sucrose utilizing a transfer reaction with β-fructofuranosidases derived from microorganisms has recently been established, they have been industrially produced. Currently, intracellular β-fructofuranosidases derived from Aspergillus niger are used in the industrial production of fructooligosaccharides.

Genes encoding these β-fructofuranosidases have been disclosed in WO 97/34004. However, these β-fructofuranosidases produce fructooligosaccharides as a mixture of 1-kestose, nystose, and 1-fructosylnystose and as a result the fructooligosaccharides have been manufactured and provided as syrup or powder of the oligosaccharide mixtures. If β-fructofuranosidases which selectively and efficiently

ATTACHMENT A

produce 1-kestose or nystose as a single component can be obtained, they would provide the following advantage. Namely, by purification of 1-kestose or nystose to a high degree, followed by crystallization, it is possible to manufacture a single-component crystal fructooligosaccharide product which has excellent characteristics in terms of properties and workability while maintaining the physiological functions of the fructooligosaccharide.

On the other hand, a method for the industrial production of crystal 1-kestose using sucrose as a raw material has been disclosed, for example, in WO 97/21718. Namely, 1-kestose is produced by reacting β-fructofuranosidase with sucrose and purified to a purity of 80% or higher by chromatographic separation, after which the resulting product is used as a crystallization material to obtain crystal 1-kestose having a purity of 95% or higher. In such a method for industrial production, characteristics of enzymes for the use required are a high conversion rate from sucrose to 1-kestose and low nystose production. Similarly, in a method for the industrial production of nystose as a single component, characteristics of the enzymes required are a high conversion rate to nystose and low 1-fructosylnystose production.

[Summary of the Invention]

An object of the present invention is to provide β-fructofuranosidase variants whose reaction specificities are improved to be suitable for the production of single components of fructooligosaccharides, such as 1-kestose and nystose, and genes of the variants.

The present inventors have found that β-fructofuranosidase variants in which amino acid residues at specific positions in the amino acid sequence of SEQ ID NO: 2 are substituted with other amino acid residues have reaction specificities suitable for the production of 1-kestose or nystose.

Namely, according to the first embodiment of the present invention, there is provided a β-fructofuranosidase variant consisting of (a) a mutated amino acid sequence of SEQ ID NO: 2, which has at least one mutation in amino acid residues at positions 62, 122, 128, 165, 221, 395, and 550 or (b) a mutated homologue of the amino acid sequence of SEQ ID NO: 2, which has at least one mutation in amino acid residues corresponding to the amino acid residues at positions 62, 122, 128, 165, 221, 395, and 550 of SEQ ID NO: 2.

According to the second embodiment of the present invention, there is provided a β-fructofuranosidase variant consisting of (c) a mutated amino acid sequence of SEQ ID NO: 2, which has at least one mutation in amino acid residues at positions 40, 379, and 381 or (d) a mutated homologue of the amino acid sequence of SEQ ID NO: 2, which has at least one mutation in amino acid residues corresponding to the amino acid residues at positions 40, 379, and 381 of SEQ ID NO: 2.

With the use of β-fructofuranosidase variants according to the present invention, it is possible to improve the sugar composition of an enzyme reaction solution upon producing fructooligosaccharides and efficiently produce single component fructooligosaccharides. Namely, β-fructofuranosidase variants according to the present invention advantageously enable the industrial production of a single component fructooligosaccharide more easily and less costly than conventional methods.

[Brief Description of the Drawings]

Figure 1 illustrates an example of the alignment of the amino acid sequence represented by SEQ ID NO: 2 and its homologues. The top line shows the amino acid sequence (SEQ ID NO: 2) of β-fructofuranosidase derived from A. niger, the middle line shows the amino acid sequence (SEQ ID NO: 6) of β-fructofuranosidase derived from S. brevicaulis, and the bottom line shows the amino acid sequence (SEQ ID NO: 4) of β-fructofuranosidase derived from P. roqueforiti. The numbers

in the Figure are amino acid numbers setting the N terminal amino acid of the A. niger-derived sequence to be 1. Specific mutation sites are shown with frames.

Figure 2 illustrates a continuation of the amino acid sequences in Figure 1.

[Detailed Description of the Invention]

β-Fructofuranosidase variants and their genes

The variants according to the first and the second embodiments of the present invention consist of mutated amino acid sequences or mutated homologues of SEQ ID NO: 2, in which a mutation is introduced into at least one specific amino acid residue.

Position numbers of the amino acid residues into which mutations are introduced correspond to the numbers of the amino acid residues of the amino acid sequence represented by SEQ ID NO: 2.

In the present invention, the term "mutation" refers to a substitution, deletion or insertion.

The term "substitution" means that a specific amino acid residue at a specific position is removed and another amino acid residue is inserted into the same position.

The term "deletion" means that a specific amino acid residue is removed.

The term "insertion" means that one or more amino acid residues are inserted before or after a specific amino acid residue, more specifically, that one or more, preferably one or several, amino acid residues are bound to an α-carboxyl group or an α-amino group of the specific amino acid residue.

The number of the specific mutations introduced into the amino acid sequence of SEQ ID NO: 2 and its homologues is not particularly limited and can be one or several, one to three, or one or two.

In the variants according to the first and the second embodiments of the present invention, the mutations introduced

into the amino acid sequence of SEQ ID NO: 2 and its homologues are preferably substitutions.

In the variants according to the first embodiment of the present invention, substitutions introduced into the amino acid residues at positions 62, 122, 128, 165, 221, 395, and 550 of the amino acid sequence of SEQ ID NO: 2 and its homologues are preferably as follows:

a substitution of the amino acid residue at position 62 with an acidic amino acid selected from the group consisting of aspartic acid and glutamic acid, in particular, with glutamic acid;

a substitution of the amino acid residue at position 122 with an amino acid selected from the group consisting of methionine, isoleucine, leucine, and valine, in particular, with methionine;

a substitution of the amino acid residue at position 128 with an amino acid selected from the group consisting of asparagine and glutamine, in particular, with asparagine;

a substitution of the amino acid residue at position 165 with an aromatic amino acid selected from the group consisting of tryptophan, phenylalanine, and tyrosine, in particular, with phenylalanine;

a substitution of the amino acid residue at position 221 with an aromatic amino acid selected from the group consisting of tryptophan, phenylalanine, and tyrosine, in particular, with tyrosine;

a substitution of the amino acid residue at position 395 with an amino acid selected from the group consisting of leucine, methionine, isoleucine, and valine, in particular, with leucine; and

a substitution of the amino acid residue at position 550 with a hydroxy amino acid selected from the group consisting of serine and threonine, in particular, with serine.

The variants according to the first embodiment of the present invention may further have mutations, preferably substitutions, in at least one amino acid residue at positions

170, 300, 313, and 386 of the amino acid sequence of SEQ ID NO: 2 and its homologues. Advantageously, β-fructofuranosidases having these mutations can selectively and efficiently produce 1-kestose (for example, see WO 99/13059).

In the variants according to the first embodiment of the present invention, substitutions which can be introduced into the amino acid residues at positions 170, 300, 313, and 386 of the amino acid sequence of SEQ ID NO: 2 and its homologues are preferably as follows:

a substitution of the amino acid residue at position 170 with an aromatic amino acid selected from the group consisting of tryptophan, phenylalanine, and tyrosine, in particular, with tryptophan;

a substitution of the amino acid residue at position 300 with an amino acid selected from the group consisting of tryptophan, phenylalanine, tyrosine, and valine, in particular, with valine;

a substitution of the amino acid residue at position 313 with a basic amino acid selected from the group consisting of lysine, arginine, and histidine, in particular, with lysine or arginine; and

a substitution of the amino acid residue at position 386 with a basic amino acid selected from the group consisting of lysine, arginine, and histidine, in particular, with lysine.

In the variants according to the first embodiment of the present invention, an example of preferred multiple mutations which can be introduced into the amino acid sequence of SEQ ID NO: 2 and its homologues is a triple mutation, preferably a triple substitution, in the amino acid residue at position 165, the amino acid residue at position 300, and the amino acid residue at position 313; in particular, a triple substitution consisting of a substitution of the amino acid residue at position 165 with an aromatic amino acid selected from the group consisting of tryptophan, phenylalanine, and tyrosine (most preferably a substitution with phenylalanine),

a substitution of the amino acid residue at position 300 with an amino acid selected from the group consisting of tryptophan, phenylalanine, tyrosine and valine (most preferably a substitution with valine), and a substitution of the amino acid residue at position 313 with a basic amino acid selected from the group consisting of lysine, arginine, and histidine (most preferably a substitution with lysine or arginine).

In the variants according to the second embodiment of the present invention, substitutions which are introduced into the amino acid residues at positions 40, 379, and 381 of the amino acid sequence of SEQ ID NO: 2 and its homologues are preferably as follows:

a substitution of the amino acid residue at position 40 with an acidic amino acid selected from the group consisting of aspartic acid and glutamic acid, in particular, with aspartic acid;

a substitution of the amino acid residue at position 379 with cysteine; and

a substitution of the amino acid residue at position 381 with an amino acid selected from the group consisting of methionine, isoleucine, leucine, and valine, in particular, with methionine.

In the variants according to the first and the second embodiments of the present invention, the term "homologue" refers to a variant of the amino acid sequence of SEQ ID NO: 2, which has one or more mutations and has β-fructofuranosidase activity. The number of mutations can be 1 to several or 1, 2, 3, or 4.

In the present invention, whether a homologue has β-fructofuranosidase activity or not can be evaluated, for example, by reacting the protein consisting of the amino acid sequence of interest with a substrate and detecting the reaction product. For example, it can be evaluated according to the method described in Example 2.

The positions of specific mutations according to the present invention in a homologue are determined based on the

position numbers of the amino acid residues in SEQ ID NO: 2 corresponding to the homologue by aligning the amino acid sequence of SEQ ID NO: 2 with said homologue. For example, "a mutation of the amino acid residue at position 62" in a homologue does not mean a mutation of the amino acid residue at position 62 of the homologue but a mutation of an amino acid residue of the homologue which corresponds to the amino acid residue at position 62 of the amino acid sequence of SEQ ID NO: 2. An example of the alignment of the amino acid sequence of SEQ ID NO: 2 and its homologues is shown in Fig. 1 and Fig. 2.

The alignment of the amino acid sequence of SEQ ID NO: 2 and its homologues can be carried out using an analytical software tool to examine the sequence homology. Such software tool is widely known and naturally can be appropriately selected for use by those skilled in the art. For example, by using the BLAST method (Basic Local Alignment Search Tool; Altschul, S.F. et al., J. Mol. Biol., 215, 403-410 (1990)), the amino acid sequence of SEQ ID NO: 2 and its homologue can be aligned to determine corresponding amino acid residues.

An example of the homologues is a variant of the amino acid sequence of SEQ ID NO: 2, which has one or more (for example, one to several, or 1, 2, 3, or 4) mutations having no effect on β-fructofuranosidase activity.

Examples of "mutations having no effect on activity" include conservative substitutions. The term "conservative substitutions" means that one or more amino acid residues are substituted with other chemically homologous amino acid residues so as not to substantially change protein activity. Examples of such substitutions include the substitution of a certain hydrophobic residue with another hydrophobic residue and the substitution of a certain polar residue with another polar residue having the same electric charge. Functionally homologous amino acids of different types which can be substituted in such a manner are known to those skilled in the art. Specific examples of such amino acids include

non-polar (hydrophobic) amino acids, such as alanine, valine, isoleucine, leucine, proline, tryptophan, phenylalanine, and methionine; polar (neutral) amino acids, such as glycine, serine, threonine, tyrosine, glutamine, asparagine, and cysteine; positively charged (basic) amino acids, such as arginine, histidine, and lysine; and further, negatively charged (acidic) amino acids, such as aspartic acid and glutamic acid.

Examples of the "homologues" include β-fructofuranosidases produced by microorganisms which belong to the genus Aspergillus, the genus Penicillium, and the genus Scopulariopsis, such as β-fructofuranosidase from Aspergillus niger, β-fructofuranosidase from Penicillium roqueforiti, and β-fructofuranosidase from Scopulariopsis brevicaulis. An example of the β-fructofuranosidase from Penicillium roqueforiti is the protein (SEQ ID NO: 4) consisting of the amino acid sequence of SEQ ID NO: 1 in WO 99/13059. An example of the β-fructofuranosidase from Scopulariopsis brevicaulis is the protein (SEQ ID NO: 6) consisting of the amino acid sequence of SEQ ID NO: 3 in WO 99/13059.

According to the present invention, there are provided genes encoding the β-fructofuranosidase variants according to the present invention.

Generally, once the amino acid sequence of a protein is provided, a DNA sequence which encodes the protein can be easily determined from the codon table. Accordingly, it is possible to appropriately select a variety of DNA sequences which encode the amino acid sequence of SEQ ID NO: 1 and its homologue into which specific mutations according to the present invention are introduced, such as the amino acid sequences of SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO: 6 into which specific mutations according to the present invention are introduced. Therefore, the DNA sequence encoding a β-fructofuranosidase variant into which specific mutations according to the present invention are introduced refers not

only to a β-fructofuranosidase gene which has DNA mutations corresponding to specific amino acid mutations according to the present invention but also to a DNA sequence which has the same DNA sequence, except that degenerate codons are used, and encodes the β-fructofuranosidase variant. For example, the DNA sequences encoding amino acid sequences of SEQ ID NO: 2, 4, and 6, into which specific mutations according to the present invention are introduced, refer not only to the DNA sequences of SEQ ID NO: 1, 3, and 5 which have one or more mutations shown in Table 3 (described later) but also to DNA sequences which have the same DNA sequences, except that degenerate codons are used, and encode β-fructofuranosidase variants.

Preparation of β-fructofuranosidase variants

A β-Fructofuranosidase variant can be prepared using recombinant DNA technology, polypeptide synthesis technology, and the like. With the use of recombinant DNA technology, DNA encoding β-fructofuranosidase (for example, the DNA sequence of SEQ ID NO: 1, 3, or 5) is obtained and site-specific mutations or random mutations are generated in this DNA to substitute amino acids to be encoded, after which a host cell is transformed with an expression vector containing the DNA treated for mutations and the resulting transformants are cultured to prepare a β-fructofuranosidase variant.

The method for the introduction of site-specific mutations into the gene can be a method known to those skilled in the art, such as the gapped duplex method and the Kunkel method. These methods can be utilized to generate mutations at specific sites of DNA encoding β-fructofuranosidase.

For the introduction of random mutations, a generally used method such as the error-prone PCR method can be used. The DNA base sequence after the mutation treatment can be confirmed by the Maxam-Gilbert chemical modification method or the dideoxynucleotide chain termination method. The amino acid sequence of the β-fructofuranosidase variant can be decoded from the confirmed nucleotide sequence.

Production of β-fructofuranosidase variants

A β-fructofuranosidase variant can be prepared by preparing a recombinant vector in which a DNA fragment encoding it is linked to a DNA molecule, in particular a DNA expression vector, which is replicable in a host cell and contains its gene in expressible conditions, introducing this recombinant vector into a host for transformation, and culturing the resulting transformants under appropriate culture conditions.

The vector used in the present invention can be appropriately selected from viruses, plasmids, cosmid vectors and the like taking the kind of host cell to be used into consideration. Examples of the vectors include pUC and pBR plasmids for Escherichia coli, pUB plasmids for Bacillus subtilis, and YEp, YRp, YCp plasmid vectors for yeasts.

According to a preferred embodiment of the present invention, a plasmid can be used as a recombinant vector. The plasmid preferably contains a selectable marker for transformation and a drug-resistance marker or a gene complementing a host auxotrophy can be used as a selectable maker. Preferred specific examples of the selectable marker include the ampicillin-resistance gene, the kanamycin-resistance gene, and the tetracycline-resistance gene for bacterial host cells; the tryptophan biosynthesis gene (TRP1), the uracyl biosynthesis gene (URA3), and the leucine biosynthesis gene (LEU2) for yeasts; and the hygromycin-resistance gene (Hyg), the bialaphos-resistance gene (Bar), and the nitrate reductase gene (niaD) for fungi.

The DNA molecule for use as an expression vector according to the present invention preferably contains nucleotide sequences necessary for the expression of a mutant gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a ribosome binding site, a translation termination signal, and a transcription termination signal.

Preferred examples of the promoter include, not to mention a promoter which is contained in an inserted fragment and can function in the host, the promoters of the lactose operon (lac) and the tryptophan operon (trp) for E. coli; the promoters of the alcohol dehydrogenase (ADH) gene, the acid phosphatase (PHO) gene, the galactose (GAL) gene, and the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene for yeasts; and the promoters of the α-amylase (amy) gene and the cellobiohydrolase I (CBHI) gene for fungi.

As a host, any cell with an established host-vector system, preferably for example, a yeast or a fungus, can be used. A transformant obtained by the transformation of the host cell is cultured under appropriate conditions and the resulting culture is subjected to a general procedure for enzyme fractionation and purification to obtain a β-fructofuranosidase variant. Further, when the host cell is Bacillus subtilis, a yeast or a fungus, a secretion vector is advantageously used to extracellularly secrete the recombinant β-fructofuranosidase.

The variant according to the present invention produced using the transformant can be obtained as follows. First, the abovementioned host cell is cultured under appropriate conditions and the culture supernatant or cells are obtained from the resultant culture using a known method such as centrifugation. The cells are further suspended in an appropriate buffer solution and then destructed by means of freezing and thawing, ultrasonication or crushing and the resulting product is centrifuged or filtered to obtain a cell extract containing the recombinant enzyme.

The enzyme can be purified by an appropriate combination of commonly used processes for separation and purification. Examples of such processes include those which utilize the difference in thermal resistance, such as heat treatment; those which utilize the difference in solubility, such as salt precipitation and solvent precipitation; those which utilize the difference in molecular weight, such as dialysis,

ultrafiltration, gel filtration, and SDS-polyacrylamide gel electrophoresis; those which utilize the difference in electric charge, such as ion exchange chromatography; those which utilize specific affinity, such as affinity chromatography; those which utilize the difference in hydrophobicity, such as hydrophobic chromatography and reversed-phase chromatography; and those which utilize the difference in isoelectric point, such as isoelectric electrophoresis.

Production of fructooligosaccharides

According to the present invention, there is provided a method for the production of fructooligosaccharides using a transformant according to the present invention or a β-fructofuranosidase variant according to the present invention. Specifically, the method for the production of fructooligosaccharides according to the present invention is carried out by bringing the transformant according to the present invention or the β-fructofuranosidase variant according to the present invention into contact with sucrose.

The mode and conditions for bringing the transformant according to the present invention or the β-fructofuranosidase variant according to the present invention into contact with sucrose are not particularly limited as long as the variant is able to act on sucrose. A preferred embodiment in which the contact proceeds in solution is as follows. Namely, the sucrose concentration can be appropriately selected within the range where the sugar to be used is soluble, taking the specific activity and reaction temperature of the enzyme into consideration. It ranges generally from 5 to 80%, preferably from 30 to 70%. The temperature and pH conditions for the reaction of the sugar and the enzyme are preferably optimized for the variant and generally range from about 30 to 80°C and from pH 4 to 10, preferably from 40 to 70°C and from pH 5 to 7.

Further, the degree of purification of the variant can be appropriately selected. The variant to be used can be a

crude enzyme from culture supernatant or crushed cells of the transformant or a purified enzyme obtained by various purification processes. Alternatively, it can be used as an isolated purified enzyme obtained by additional various purification processes.

Furthermore, the enzyme can be brought into contact with sucrose in an immobilized form onto a carrier according to an ordinary method.

Fructooligosaccharides can be purified from the resulting reaction solution according to a known method. For example, the solution is heated to inactivate the enzyme, decolorized using activated carbon, and then desalted using ion exchange resins.

When the variant of the first embodiment of the present invention is used for the preparation of fructooligosaccharides, the production of 1-kestose is increased and the production of nystose is decreased. Therefore, according to the present invention, there is provided a method for the selective production of 1-kestose. Namely, according to the present invention, there is provided a method for the production of 1-kestose, comprising the step of bringing the β-fructofuranosidase variant of the first embodiment or a transformant which can express a polynucleotide encoding the β-fructofuranosidase variant of the first embodiment into contact with sucrose.

When the variant of the second embodiment of the present invention is used for the preparation of fructooligosaccharides, the production of nystose is increased and the production of 1-kestose is decreased. Therefore, according to the present invention, there is provided a method for the selective production of nystose. Namely, according to the present invention, there is provided a method for the production of nystose, comprising the step of bringing the β-fructofuranosidase variant of the second embodiment or a transformant which can express a polynucleotide

encoding the β-fructofuranosidase variant of the second embodiment into contact with sucrose.

[Examples]

The present invention will be illustrated more in detail with reference to the following examples; however, these examples are not construed to limit the scope of the invention.

Example 1: Preparation of β-fructofuranosidase variants

Introduction of random mutations into the β-fructofuranosidase gene was carried out using a commercially available PCR mutagenesis kit (Gene Morph, Stratagene) as follows. The β-fructofuranosidase gene from the ATCC 20611 strain (A. niger) was used as a template DNA. Specifically, plasmid pAW20-Hyg described in WO 97/34004 was used. A PCR reaction solution contained 1 μl of template DNA, 1 μl of 40 mM dNTP, 5 μl of a 10-fold concentrated buffer solution, 0.5 μl each of 250 ng/ml 5'-GCGAATTCATGAAGCTCACCACTACCA-3' (N-terminal) (SEQ ID NO: 7) and 5'-GCGGATCCCGGTCAATTTCTCT-3' (C-terminal) (SEQ ID NO: 8) as primers, 1 μl of Mutazyme, 5 μl of DMSO, and 36 μl of sterile water to make the total volume 50 μl. The reaction was carried out by 30 cycles of incubation at 94°C for 1 minute (denaturation step), at 50°C for 2 minutes (annealing step), and at 72°C for 2.5 minutes (elongation step), after pretreatment at 94°C for 2 minutes. Finally, incubation was carried out at 72°C for 3 minutes to complete the reaction. The reaction solution was subjected to extraction with phenol/chloroform/isoamyl alcohol and then precipitation with ethanol. The precipitate was dissolved in a TE buffer solution, after which the resulting solution was subjected to agarose gel electrophoresis and a specifically amplified band of 1.9 kbp was excised to recover a DNA fragment according to an ordinary method. A plasmid in which the 1.9 kbp EcoRI-BamHI fragment was inserted into the EcoRI-BamHI site of pY2831 was introduced into the S. cerevisiae MS-161 strain by the lithium acetate method to obtain a transformant, according to the method described in WO 97/34004. The transformant thus obtained was cultured in an SD-GF medium (0.67% yeast nitrogen base

without amino acids, 2% sucrose, 2% casamino acids, and 50 μg/ml uracyl) at 30°C for 3 days to obtain a β-fructofuranosidase variant.

Example 2: Evaluation of reaction specificity of β-fructofuranosidase variants

The enzyme reaction was carried out at pH 7 at 40°C using the β-fructofuranosidase variants prepared in Example 1 and sucrose as a substrate at a substrate concentration of 48% and the sugar composition of each resulting reaction solution was subjected to HPLC analysis. The sugar composition of each enzyme reaction solution was compared with the sugar composition with the wild-type β-fructofuranosidase and variants showing altered compositions were selected as β-fructofuranosidase variants with altered reaction specificity.

In order to identify mutation points of the β-fructofuranosidase variants with altered reaction specificity, DNA base sequences were analyzed. Sequencing reaction was carried out using a DNA sequencing kit by Pharmacia. Samples after the reaction were analyzed using DNA sequencer (ALFred) by Pharmacia to obtain base sequences of individual DNA fragments. Then, final base sequences were obtained using a DNA analysis software (DNASIS, Hitachi Software Engineering) to determine the mutation points into which random mutations were introduced. As a result, as shown in Table 1 and Table 2, it was revealed that β-fructofuranosidase variants which efficiently produce 1-kestose and β-fructofuranosidase variants which efficiently produce nystose were obtained.

Table 1: β-fructofuranosidase variants with which 1-kestose production is increased and nystose production is decreased

	F	G	GF	GF_2	GF_3	GF_4
Wild-type	0.4	22.3	20.5	45.1	11.3	0.3
G62E	0.6	22.1	21.1	46.0	10.0	0.2
L122M	0.7	22.1	19.7	47.9	9.6	0.0
I128N	0.8	20.7	26.5	45.1	6.5	0.5
V165F	0.6	22.0	19.8	46.8	10.8	0.0
H221Y	0.6	23.8	20.1	45.8	9.5	0.2
Q395L	0.6	22.1	21.4	46.5	9.1	0.2
T550S	0.9	26.3	13.1	48.4	10.4	0.9

F: Fructose

G: Glucose

GF: Sucrose

GF2: 1-Kestose

GF3: Nystose

GF4: 1-Fructosylnystose

Table 2: β-fructofuranosidase variants with which nystose production is increased and 1-kestose production is decreased

	F	G	GF	GF_2	GF_3	GF_4
Wild-type	0.4	22.3	20.5	45.1	11.3	0.3
G40D	0.6	22.3	20.3	41.6	14.7	0.5
T381M	1.5	23.7	23.9	28.8	19.3	2.8
W379C	1.1	22.6	22.5	36.2	17.0	0.6

F: Fructose

G: Glucose

GF: Sucrose

GF2: 1-Kestose

GF3: Nystose

GF4: 1-Fructosylnystose

Mutations obtained and their corresponding DNA sequences are as follows. Underlined are mutated DNAs.

Table 3: Amino acid residues and DNA sequences at mutation sites

G62E	GAC	GAG	GAC	
	Asp	Glu	Asp	(SEQ ID NO: 9)
L122M	TTC	ATG	CCC	
	Phe	Met	Pro	(SEQ ID NO: 10)
I128N	TCC	AAC	CCC	
	Ser	Asn	Pro	(SEQ ID NO: 11)
V165F	GCC	TTC	GAC	
	Ala	Phe	Asp	(SEQ ID NO: 12)
H221Y	GTG	TAC	GGC	
	Val	Tyr	Gly	(SEQ ID NO: 13)
Q395L	GCC	CTG	CAG	
	Ala	Leu	Gln	(SEQ ID NO: 14)
T550S	TTT	TCG	GAG	
	Phe	Ser	Glu	(SEQ ID NO: 15)
G40D	ATC	GAC	GAC	
	Ile	Asp	Asp	(SEQ ID NO: 16)
T381M	TTG	ATG	GGC	
	Leu	Met	Gly	(SEQ ID NO: 17)
W379C	GTC	TGC	TTG	
	Val	Cys	Leu	(SEQ ID NO: 18)

Example 3: Preparation of multiple substitution variants by site-directed mutations and evaluation of their reaction specificity

A ternary substitution variant was prepared by introducing site-directed mutations using the combination of V165F obtained in Example 2 and G300V and H313K described in WO 97/34004. Specifically, the β-fructofuranosidase gene prepared in Examples 1 and 2, into which the mutation V165F was introduced, was inserted into the EcoRI-BamHI site of pUC118 (Takara Shuzo) to prepare a plasmid. Next, the mutations G300V and H313K were introduced one by one in the same manner

as described in Example D8 in WO 97/34004. The DNA base sequences were examined in the same manner as in Example 2, which confirmed that the base sequences at the site of interest only were substituted.

The reaction specificity of the ternary substitution variant V165F + G300V + H313K was examined according to the method in Example 2. The result is shown in Table 3. The 1-kestose production was increased about 10% and the nystose production was decreased 7% with the ternary substitution variant as compared to those with the wild-type β-fructofuranosidase.

Table 4: Reaction specificity of ternary substitution variant

	F	G	GF	GF_2	GF_3	GF_4
Wild-type	0.4	22.3	20.5	45.1	11.3	0.3
V165F/G300V/H313K	1.7	22.5	15.8	55.7	4.3	0.0

F: Fructose
G: Glucose
GF: Sucrose
GF2: 1-Kestose
GF3: Nystose
GF4: 1-Fructosylnystose